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Full Length Research Paper

Influence of pretreatment of agriculture residues on phytase production by *Aspergillus niger* NCIM 563 under submerged fermentation conditions

Kavita Bhavsar, Parin Shah, Sarvesh Kumar Soni and J. M. Khire*

NCIM Resource Center, National Chemical Laboratory, Pune 411 008, India.

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The extracellular phytase production by *Aspergillus niger* NCIM 563 was evaluated in medium containing various agriculture residues under submerged fermentation conditions. Phytase production was affected by inorganic phosphate content of agriculture residues which ranged from 2.8 to 8 mg/g. The agriculture residues containing less than 4 mg/g inorganic phosphate supported phytase production with maximum activity of 68 IU/ml in medium containing 1% rice bran on 11th day of fermentation. Addition of glucose up to 5% in fermentation medium containing 1% rice bran, enhanced phytase production. Pretreatment of agriculture residues with water to remove excess inorganic phosphate has significantly enhanced the phytase activity in case of de-oiled rice bran, wheat bran, peanut cake (low and high oil) and coconut cake. Maximum increase of 20.3 times in phytase activity was observed in case of wheat bran as compared to de-oiled rice bran, coconut cake, peanut cake high and low oil in which the increase in phytase activity was 6.85, 6.1, 5.3 and 3.0 times, respectively. Maximum phytase activity of 68 IU/ml was produced on the 11th day of fermentation compared to earlier reported 41.47 IU/ml phytase activity on the 15th day of submerged fermentation using 5% dextrin and 2.5% glucose, thus increasing productivity.

Key words: Phytase, *Aspergillus niger*, submerged fermentation, phytate degradation, poultry feed supplement, agriculture residue.

INTRODUCTION

Phytic acid (myo-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate) is a major storage form of phosphorus and the source of inositol in plant seeds (Reddy et al., 1982; Dvorakova 1998). Phytases (EC 3.1.3.8 for 3-phytase and EC 3.1.3.26 for 6-phytase) hydrolyses phytic acid to myo-inositol and phosphoric acid in a stepwise manner forming myo-inositol phosphate intermediates (Mullaney et al., 2000). Since phytase is either absent or present at a very low level in the gastrointestinal tract of monogastric animals (Jongbloed et al., 1992; Maenz and Classen, 1998; Selle and Ravindran, 2007) dietary phytate is not digested in the intestine and consequently accumulates in fecal materials. Phytate thus contributes to phosphorus pollution in areas of intensive animal production. Due to its strong chelating property phytic acid is regarded as an anti-nutritive factor because it forms insoluble complexes with nutritionally important minerals such as calcium, zinc, magnesium and iron, decreasing their bioavailability (Erdman and Poneros-Schneier, 1989; Fox and Tao, 1989). Phytase has been used as feed supplement to improve phosphorus nutrition and reduce phosphorus in excretory products of animals (Ravindran et al., 2001).

There are various reports on phytase production by bacteria, yeast and fungi (Vohra and Satyanarayana, 2003; Vats and Banerjee, 2004). Among them, strains of *Aspergillus niger* (syn. *A. ficuum*) produce large amounts of extracellular phytase (Chelius and Wodzinski, 1994) and show more acid tolerance than bacteria and yeasts (Kim et al., 1998). In view of its industrial importance the

*Corresponding author. Email: jmkhire@yahoo.com or jm.khire@ncl.res.in. Tel: 091-020-25902452. Fax: 091-020-25882647

ultimate objective is to produce this enzyme at cost effective level and establish conditions for its industrial application. In the present communication we report enhanced phytase production under submerged fermentation conditions using pretreated agriculture residues to remove excess inorganic phosphate which otherwise inhibit phytase production. We report a simple pretreatment of agriculture residue for phytase production and for enhanced phytase activity. Earlier we have reported phytase production by solid-state fermentation (SSF) of agriculture residue using *A. niger* NCIM 563 which was highly active at pH 5.0 (Mandviwala and Khire, 2000) and process for preparation of acidic phytase using dextrin-glucose medium under submerged fermentation condition (Soni and Khire, 2005; Soni and Khire, 2007).

MATERIALS AND METHOD

Chemicals

Phytic acid sodium salt was purchased from Sigma Chemical Company, St Louise, MO, USA. All other chemicals used were of analytical grade and obtained from leading manufacturers including Sigma, BDH and Glaxo.

Fungal strain

A. niger NCIM 563 was used in the present study from NCIM Resource Center, Pune, India. The stock cultures were maintained on Potato Dextrose Agar (PDA) slants and stored at 4°C. Spores for inoculation were obtained by culturing the strain at 30°C on PDA slants for 7 days, followed by washing with 10 ml sterile distilled water containing 0.01% Tween 80.

Agriculture residues

Various agriculture residues such as rice bran, de-oiled rice bran, wheat bran, maize, chickpea, peanut cake (low oil), peanut cake (high oil) and coconut cake were purchased from a local market.

Inorganic phosphate content of agriculture residue

Initial inorganic phosphate content of each agriculture residue was determined by suspending 1 g of residue in 100 ml distilled water for 30 min, centrifuged to remove supernatant and dried in oven at 60°C before use. The liberated inorganic phosphate in the supernatant was determined by a modification of the ammonium molybdate method (Heinohen and Lathi, 1981).

Reduction of inorganic phosphate from agriculture residues

For reduction of inorganic phosphate content residues were treated by two methods. In the first method (Sano et al., 1999) 10 g agriculture residue was suspended in 100 ml of 10 mM $MgCl_2$ pH 8.5 (with 2M KOH) and kept at 4°C for 24 h. The supernatant was removed by centrifugation at 8000 g for 15 min and residue was dried in oven before use. In second method 1 g agriculture residue was suspended in 100 ml distilled water. Supernatant was removed by centrifugation and residue was oven dried before use and checked for phosphate content (Heinohen and Lathi, 1981).

Medium and culture conditions

Fermentation medium for phytase production was according to Shieh and Ware (1968) with few modifications. Soluble starch was replaced with agriculture residue and inorganic phosphorus source was omitted. Thus modified fermentation medium contained (per 100 ml): rice bran 1 g; glucose 5 g; $NaNO_3$ 0.86 g; KCl 0.05 g; $MgSO_4.7H_2O$ 0.05 g; $FeSO_4.7H_2O$ 0.01 g, and pH 5.5 before sterilization.

Fermentation medium (100 ml in 250 ml Erlenmeyer flask) was inoculated with 1% (v/v) of spore suspension (5 x 10^7 spores per ml) prepared by suspending the spores from 7 day old sporulated slant of *A. niger* NCIM 563 grown on PDA in 10 ml of sterile distilled water containing 0.01% (v/v) Tween 80 and incubated at 30°C at 200 rpm. Samples were removed after every 24 h and checked for pH, growth, total residual reducing sugar, extra cellular protein and phytase activity. Various agriculture residues and glucose conc. were used in the fermentation medium to study their effect on production of phytase.

Phytase assay

Phytase activity was measured at 50°C as described earlier (Mandviwala and Khire, 2000). The reaction was carried out at pH 2.5 using 100 mM Glycine-HCl buffer at 50°C for 30 min. The liberated inorganic phosphate was measured by a modification of the ammonium molybdate method (Heinohen and Lathi, 1981). A freshly prepared four ml solution of acetone: 5 N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v/v) and 400 µl of 1 M citric acid were added to the assay mixture. Absorbance was measured at 370 nm. One unit of phytase activity (IU) was expressed as the amount of enzyme that liberates 1 µmole phosphorus per minute under standard assay conditions. Each experiment was carried out in triplicate and the values reported are the mean of three such experiments in which a maximum of 3 - 5% variability was observed.

Protein estimation

Protein concentration in the culture filtrate was determined by the method of Lowry et al. (1951), using Bovine serum albumin as a standard. The biomass was measured after drying at 105°C for 24 h.

Sugar content

Total residual reducing sugar concentration was estimated by DNSA method (Miller 1959) and HPLC system (Dionex India Limited, Mumbai, India) equipped with UV- or RI-detectors. An ion exclusion column (Aminex HPX-87H; Bio-Rad, Hercules, CA, USA) was used at a temperature of 38°C with 8 mM H_2SO_4 as a mobile phase at a flow rate of 6 ml/min.

RESULTS AND DISCUSSION

During the past decade, the addition of microbial phytase in poultry diets has increased remarkably with substantial documented evidence of its role in release of phytate-bound phosphorus and reduction of otherwise undigested phosphorus in excreta (Selle and Ravindran, 2007). Mondal et al. (2007) has shown that supplementation of microbial phytase in soybean meal based broiler diets

Table 1. Initial phosphate content in various agriculture residues.

Agriculture residue	Phosphate concentration (mg/g)	
	Before treatment	After treatment
Rice bran	2.7	0.47
De – oiled rice bran	4.0	1.08
Wheat bran	4.02	0.56
Peanut cake (low oil)	4.95	2.9
Peanut cake (high oil)	5.89	2.7
Chickpea	2.8	0.95
Maize	2.3	2.2
Coconut cake	8.0	1.43

The values given in the table are the average of three independent experiments with 3 - 5% variation.

containing low phosphorus has increased the retention of Ca and P with compensation of the untoward effect of low phosphorus levels from the diet. Similarly, Panda et al. (2007) has also shown that supplementation of microbial phytase to low non phytate phosphorus diets for broiler chickens improved bone mineralization and retention of nutrients. Only the cost of microbial phytase production should be economical to justify its application in poultry feed. In the present communication we report simple pretreatment of agriculture residues for increased phytase activity under submerged fermentation conditions.

Initial phosphate concentration of various agriculture residues and effect of pretreatment

Preliminary results indicate that initial inorganic phosphate concentration of various agriculture residues ranges from 2.30 mg/g to 8.0 mg/g (Table 1). Treatment of agriculture residues suspended in distilled water with magnesium chloride precipitates the phosphate**,** so the supernatant becomes free of inorganic phosphate. This method is useful for the removal of phosphate from liquid medium like diluted molasses (Sano et al., 1999) but can not be used for removal of phosphate from insoluble material like agriculture residue, as precipitate of inorganic phosphate adheres to the agriculture residue. On the other hand, considerable amount of inorganic phosphate was removed when agriculture residues were suspended in distilled water (Table 1). *A. niger* NCIM 563 grew well and produced phytase in modified basal medium (100 ml in 250 ml flask) containing 1 g rice bran and 5.0 g glucose as carbon source.

Time course of phytase production with untreated agriculture residue

The time course of phytase production under submerged fermentation conditions in medium (100 ml in 250 ml flask) containing 1 g agriculture residue and 5.0 g glucose and inorganic salts is shown in Table 2a. The fungus grew rapidly as indicated in all the agriculture residues with rapid utilization of glucose. Maximum phytase activity was secreted at around 10 to 11th day. Agriculture residues containing less than 4 mg/g inorganic phosphate supported phytase production with maximum activity of 68 IU/ml in rice bran containing medium followed by chickpea and maize (58 and 53.2 IU/ml, respectively). Agriculture residues containing inorganic phosphate more than 4 mg/g supported the growth of the fungus but phytase activity was very low. Earlier we have reported inhibition of phytase secretion in medium containing more than 4 mg/100 ml medium in dextrin-glucose medium. Various reports established that the phytase production in submerged and solid state fermentation is affected by the amount of inorganic phosphorus in fermentation medium (Shieh and Ware, 1968; Reddy et al., 1982; Dvorakova 1998; Mullaney et al., 2000; Vohra and Satyanarayana, 2003). High levels of inorganic phosphorus repress the biosynthesis of phytase. Thus soluble inorganic phosphate in the agriculture residue plays very important role in phytase secretion.

Time course of phytase production with pretreated agriculture residue

Time course of phytase production in medium (100 ml in 250 ml flask) containing 1 g pretreated agriculture residue and 5.0 g glucose and inorganic salts is shown in Table 2b. Pretreatment of agriculture residues with water to remove excess inorganic phosphate has significantly enhanced the phytase activity in case of de-oiled rice bran, wheat bran, peanut cake (low and high oil) and coconut cake. Maximum increase of 20.3 times in phytase activity was observed in case of wheat bran as compared to de-oiled rice bran, coconut cake, peanut cake high and low oil where in the increase in phytase activity was 6.85, 6.1, 5.3 and 3.0 times, respectively. In case of rice bran and chickpea, the pretreatment with water resulted in 75% decrease in phytase activity which can be co-related to reduction of inorganic phosphate content from 2.7 to 0.47 mg/g in case of rice bran and 2.8 to 0.95 mg/g for chickpea. In case of maize decrease in phytase activity was only 25% (Figure 1).

Effect of glucose on phytase production

The effect of easily metabolizable sugar, glucose (1 - 9% w/v) on phytase production, in 1% rice bran fermentation medium indicates that gradual increase in phytase activity was observed when sugar concentration in the fermentation medium was increased from 1 to 5%. Maximum activity of 68 IU/ml was produced in medium containing 5% glucose. Sugar utilization was very rapid

Table 2a. Time course of phytase production using agriculture residues before pretreatment.

Agriculture residues	Phytase activity (IU/ml) on day				
	4	7	10	11	12
Rice bran	14.5	44	56.2	68	60.5
De-oiled rice bran	3.0	5.2	7.0	0.74	0.50
Wheat bran	1.9	2.6	2.6	2.2	2.0
Peanut cake (low oil)	4.15	7.3	9.3	8.9	5.6
Peanut cake (high oil)	3.1	5.2	6.0	6.5	4.2
Chickpea	14.9	40	59	58	53
Maize	14.7	35	49	53.2	49.5
Coconut cake	2.0	3.0	4.0	3.7	2.5

The values given in the table are the average of three independent experiments with 3 - 5% variation.

Table 2b. Time course of phytase production using agriculture residues after pretreatment.

Agriculture residues	Phytase activity (IU/ml) on day				
	4	7	10	11	12
Rice bran	3.9	9.0	13.6	16.1	14.0
De-oiled rice bran	9.0	31.0	47.0	48.0	42.0
Wheat bran	9.0	39.3	45.4	52.9	49.2
Peanut cake (low oil)	7.5	14.0	26.6	28.5	20.6
Peanut cake (high oil)	6.6	22.0	34.2	27.8	20.7
Chickpea	6.0	12.0	18.3	19.4	15.4
Maize	10.8	25.0	38.0	40.5	35.6
Coconut cake	6.5	12.6	23.4	24.4	20.4

The values given in the table are the average of three independent experiments with 3 - 5% variation.

Table 3. Phytase production at different glucose concentration using 1% rice bran.

Days	Glucose concentration in fermentation medium									
	1%		3%		5%		7%		9%	
	Activity (IU/ml)	Reducing sugar (%)	Activity (IU/ml)	Reducing sugar (%)	Activity (IU/ml)	Reducing sugar (%)	Activity (IU/ml)	Reducing sugar (%)	Activity (IU/ml)	Reducing sugar (%)
2	0.94	0.13	1.0	2.2	0.83	4.4	1.06	6.8	1.35	8.9
4	5.7	0.025	12.5	0.073	12.0	1.9	10.4	3.46	8.8	5.82
7	6.0	0.012	21.0	0.02	36.0	0.19	33.0	2.0	30.0	4.36
9	7.5	0.014	35.0	0.023	58.0	0.059	58.5	1.31	50.5	2.71
11	7.2	0.019	38.0	0.003	68.0	0.019	61.0	0.59	56.0	0.76
12	6.5	0.007	33.0	0.002	58.0	0.011	48.0	0.45	45.0	0.50

The values given in the table are the average of three independent experiments with 3 - 5% variation.

and maximum phytase activity was correlated with minimum reducing sugar in fermentation medium (Table 3). Easily metabolizable sugar e.g. glucose has been reported to increase phytase production by *A. niger* during submerged and/or solid-state fermentation (Vats and Banerjee, 2002; Vats and Banerjee, 2004; Vats et al., 2004).

Effect of rice bran concentration production of phytase

The effect of various concentration of rice bran (0.5 to 2%) in fermentation medium containing 5% glucose, indicates that maximum phytase activity was produced in

Table 4. Comparison of phytase activity in Dextrin-glucose and rice bran-glucose medium.

Fermentation time (days)	1% Rice bran and 5% Glucose medium		5 % Dextrin and 2.5% Glucose medium	
	Activity (IU/ml)	Reducing sugar (%)	Activity (IU/ml)	Reducing sugar (%)
4	12	1.9	4.17	5.26
7	36	0.19	10	4.3
9	58	0.05	16	3.5
11	68	0.019	23.5	2.6
12	62	0.018	26	2.28
14	59	0.010	31.68	1.75
15	51	0.009	41.47	1.03
16	50	0.009	37	0.91

The culture was grown under submerged fermentation condition at 30^0C with shaking (200 rpm) as described in Material and Methods .The values given in the table are the average of three independent experiments with 3 - 5% variation.

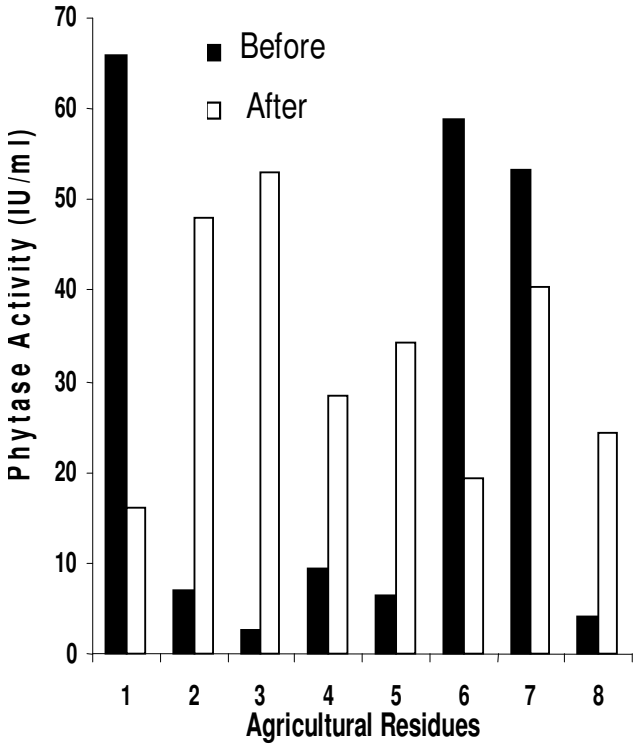

Figure 1. Comparison of phytase activity before and after pretreatment of agriculture residue.
1 = Rice bran; 2 = de =oil rice bran; 3 = wheat Bran; 4 = peanut cake (low oil); 5 = peanut cake (high oil); 6 = chickpea; 7 = Maize; and 8 = coconut cake

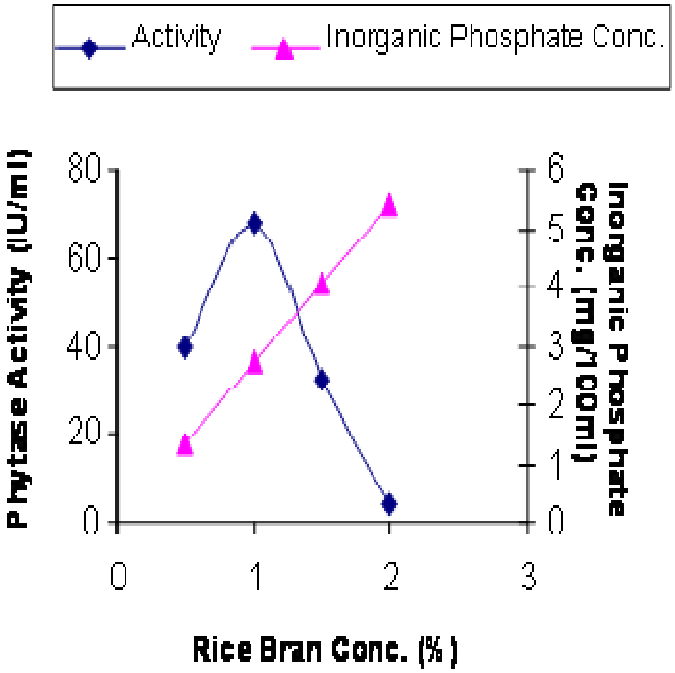

Figure 2. Effect of various concentration of rice bran on phytase production

medium containing 1% rice bran i.e. the medium containing 2.7 mg inorganic phosphate per 100 ml medium (Figure 2). Increasing the concentration of rice bran above 1% concentration resulted into more inorganic phosphate in the medium which resulted in inhibition of phytase activity. Similarly, medium containing 0.5% rice bran resulted in less phytase activity due to insufficient inorganic phosphate in the medium.

Comparison of phytase production in dextrin-glucose and rice bran-glucose medium

Earlier we have reported phytase production under submerged fermentation medium containing 5% dextrin and 2.5% glucose, in which maximum phytase activity of 41.47 IU/ml was produced on 15^{th} day of fermentation medium (Soni and Khire, 2005, 2007). In the present work medium containing 1% rice bran and 5% glucose gave maximum phytase activity of 68 IU/ml was produced within 11^{th} day only (Table 4). Thus fermentation time was reduced by four days with increase in activity by 70%.

Conclusion

Present studies on phytase production under submerged fermentation conditions by *A. niger* NCIM 563, indicates that pretreatment of agriculture residues with distilled water was useful when initial inorganic phosphate content of the residue was above 4 mg/g, which otherwise inhibit phytase production. There was substantial increase in phytase activity when this excess phosphate was removed by pretreatment. Maximum increase (20.3 times) in phytase activity was observed in case of wheat bran. However pretreatment was not useful in case of rice bran where initial inorganic phosphate content was 2.7 mg/g which was reduced to 0.47 mg/g after washing the residue with distilled water which resulted in decrease in phytase activity from 68 IU/ml (before treatment) to 16.1 IU/ml (after treatment). Similarly there was increase in productivity and reduction in fermentation time when agriculture residue was used instead of dextrin in submerged fermentation. Maximum phytase activity of 68 IU/ml was produced on the 11th day of fermentation compared to 41.47 IU/ml activity on the 15th day of submerged fermentation using 5% dextrin and 2.5% glucose. Similarly, cost of any agriculture residue is much cheaper than dextrin. Further experiments in up scaling the process at 10 L fermenter scale and application of phytase in poultry feed are in progress.

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